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THE BINDING OF HEMOGLOBIN TO MEMBRANES OF NORMAL AND SICKLE ERYTHROCYTES

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SUMMARY

The binding of hemoglobins A, S, and A_2 to red cell membranes prepared by hypotonic lysis from normal blood and blood from persons with sickle cell anemia was quantified under a variety of conditions using hemoglobin labelled by alkylation with ^{14}C-labelled Nitrogen Mustard. Membrane morphology was examined by electron microscopy. Normal membranes were found capable of binding native hemoglobin A and hemoglobin S in similar amounts when incubated at low hemoglobin: membrane ratios, but at high ratios hemoglobin saturation levels of the membranes increased progressively for hemoglobin A, hemoglobin S and hemoglobin A_2, respectively, in order of increasing electropositivity. Binding was unaffected by variations in temperature (4–22 °C) and altered little by the presence of sulfhydryl reagents, but was inhibited at pH levels above 7.35; disrupted at high ionic strength; and dependent on the ionic composition of the media. These findings suggest that electrostatic, but not hydrophobic or sulfhydryl bonds are important in membrane binding of the hemoglobin under the conditions studied.

An increased retention of hemoglobin in preparations of membranes from red cells of patients with sickle cell anemia (homozygote S) was attributable to the dense fraction of homozygote S red cells rich in irreversibly sickled cells, and the latter membranes had a smaller residual binding capacity for new hemoglobin. This suggests that in homozygote S cells which have become irreversibly sickled cells in vivo, there are membrane changes which involve alteration and/or blockade of hemoglobin binding sites.

These findings support the notion that hemoglobin participates in the dynamic structure of the red cell membrane in a manner which differs in normal and pathological states.

INTRODUCTION

The relationship between hemoglobin and the erythrocyte membrane has concerned many investigators, who noted the retention of various amounts of hemoglobin in preparations of red cell ghosts. The question of whether or not hemoglobin

is a "structural" component of these membranes was formerly judged on the basis of the relative ease of removal of the hemoglobin and the functional and morphological status and chemical composition of the various ghost preparations. For example, the ghosts prepared by multiple washes in distilled water retained 3 % of the total hemoglobin of the original cells, so that hemoglobin represented about half of the dry weight of the membrane preparations [1]. In contrast, the ghosts prepared by gradual osmotic lysis contained only 1.4 % (dry weight) hemoglobin, showed no loss of cholesterol or phospholipid, and retained biconcave disc shape and osmotic responsiveness [2]. Other studies demonstrated that the extent of removal of hemoglobin (and probably of various membrane proteins as well) varied with the pH and ionic strength of the hemolyzing solutions and suggested that hemoglobin was attached to the membranes by non-covalent bonds [3, 4].

More recent, dynamic views of the plasma membrane make a less distinct division between the membrane and the soluble interior of the cell [5], and the relationship between hemoglobin and the red cell membrane is now considered in terms of the possible role of hemoglobin in the functional integrity of the membrane. It has been shown that membranes may include some insoluble hemoglobin in ATP-depleted cells or in cells containing Heinz bodies [6]. Little information, however, exists as to whether or not membranes washed nearly free of hemoglobin are capable of binding native hemoglobin [4]. As it can be readily seen, the previous work in this field has dealt basically with the description and conditions that modified the retention of hemoglobin by red cell membrane preparations.

The present studies were designed to determine the ability of red cell membranes to bind native hemoglobin. A system is described in which such binding can be directly observed and measured. Following initial binding experiments using normal membranes and hemoglobin A, the hemoglobin binding properties of membranes from patients with sickle cell anemia were studied to explore the possible relationship between membrane binding of hemoglobin S and the red cell deformities in this disorder.

MATERIALS AND METHODS

Preparation of red cell membranes

Red cells from freshly drawn blood were washed three times in 0.15 M NaCl buffered with 5 mM potassium phosphate, pH 7.4, and the membranes prepared by hypotonic lysis [7]. A proportion of lysing buffer to cells of 50 to 1 was used for each wash. Similar results were obtained using ghosts prepared by the gradual osmotic lysis procedure of Danon et al. [8]. In this procedure lysis was accomplished by supending the cells in an isotonic solution of 60 mM NaCl, 25 mM Na_2HPO_4 and 5 mM KH_2PO_4, pH 7.2, and then dialyzing against the same buffer and pH but lowering the osmolarity to 30 mosM and adding 1 mM EDTA. Membranes were collected by centrifugation at 37 000 $\times g$ for 15 min, washed in 30 mM NaCl, 5 mM Tris and 1 mM EDTA, pH 7.2, for 5 times. In both procedures the membranes were finally suspended in 100 mM Tris–HCl buffer, pH 7.4, at 4 °C.

Membranes were prepared from red cells of patients with sickle cell anemia (homozygote S) by the above procedure with the following modifications. The homozygote S red cells were first oxygenated for 3 min and then fractionated as

follows: The washed cells (hematocrit adjusted to 60 % with the buffered saline) were centrifuged at 140 000 × *g* for 60 min at 20 °C (Beckman Model RC2, SW-65 rotor) and three fractions of cells separated as described by Bertles and Milner [9]. The upper portion of cells (Fraction III) and the middle fraction (Fraction II) were removed by pipette. After the centrifuge tube was tilted and allowed to drain, a residual fraction (I) adhered to the tube; this fraction which contained a high proportion of "irreversibly sickled cells", was removed from the tube with 5 mM potassium phosphate buffer, pH 8.0. In some experiments the washed oxygenated homozygote S red cells were suspended in autologous plasma (at 60 % hematocrit) before centrifugation. In this case, there was adequate separation of the three cell fractions, but the bottom fraction had to be removed by pipette, resulting in decreased recovery of the fraction. The membranes prepared from normal blood and from Fractions II and III of homozygote S blood appeared white and were nearly free of hemoglobin, whereas those prepared from Fraction I of homozygote S blood were slightly pink. Several samples of centrifuged homozygote S cells were examined microscopically after fixation in 10 % formalin in 0.9 % NaCl. The proportion of irreversible sickle cells (elongated or sickled) was about 50 % in Fraction I, and decreased sharply in the upper fractions (Fractions II and III).

Hemoglobin preparation

Hemolysates were prepared according to Drabkin [10] from normal and from homozygote S blood. Purification of hemoglobin A_2 was obtained from a normal hemolysate by DE-52 cellulose chromatography, developed with a 0.05 M Tris–HCl buffer, pH 8.0. A breakable column of 15 cm internal diameter was used. Once hemoglobin A_2 had travelled to the lower portion of the column, the upper portion was removed, and the hemoglobin A_2 was eluted by 0.5 M NaCl added to the developing buffer.

Hemolysates from homozygote A and homozygote S individuals were not purified any further. Patients with sickle cell anemia were selected for having a low percentage of Hb F (less than 6 %).

Radioactive labelling of hemoglobin

Hemolysates or hemoglobin solutions were dialyzed overnight at 4 °C against 0.15 M potassium phosphate buffer, pH 7.35. ^{14}C-labelled bis-β-chloroethylmethylamine hydrochloride (nitrogen mustard, HN2) (Mallinckrodt Co., St. Louis, Mo.) with a specific activity of 100 μCi per 2.62 mg was dissolved in the same buffer and allowed to react with lysate for 30 min at room temperature as described by Roth et al. [11]. The hemoglobin solution was then dialyzed against three changes of the buffer for 48 h at 4° C. Hemoglobin labelled in such a manner has been shown to attain constant specific activity after 24 h of dialysis, which usually results in the loss of about 85 % of the total radioactivity.

Incubation of membrane with labelled hemoglobin

Samples of membrane preparations containing known quantities of membrane protein were mixed with labelled hemoglobin so that the ratio of membrane protein to hemoglobin was between 1.5:1 and 2.5:1. The mixtures were incubated for various

time periods between 10 and 120 min at 4 or at 22 °C, in Tris–HCl buffer of varying pH, ionic strength, and ionic composition, as specified in Tables I and II.

In certain experiments, membranes were first incubated with unlabelled hemoglobin for 30 min at 22 °C, freed of unbound hemoglobin as described below, and then resuspended in the incubation media together with labelled hemoglobin.

Separation of free hemoglobin from membranes with bound hemoglobin

Following incubation, samples were layered on pre-equilibrated linear sucrose gradients (between 15 and 60 % sucrose in 10 mM Tris–HCl, pH 7.2, containing 5 mM Mg^{2+}) and centrifuged at 96 000 × *g* for 90 min at 4 °C in an SW-27 rotor. This procedure resulted in a clear separation of free hemoglobin from hemoglobin bound to membranes. Aliquots were drawn from the bottom of the tube through a needle inserted from the top and transferred by a peristaltic pump to a Gilford Model 240 recording spectrophotometer. 34 fractions were collected from each sample and the proteins, precipitated with 20 % trichloroacetic acid, were collected on Whatman glass filter pads, washed with 5 % trichloroacetic acid, dried and the radioactivity determined with a Packard Tricarb liquid scintillation counter (overall efficiency of 77 % for ^{14}C). Since the specific activity of the added hemoglobin was known, the quantity of hemoglobin bound to membrane protein in each fraction could be calculated.

As an alternative separation procedure, the incubated suspension was centrifuged at 145 000 × *g* for 60 min at 4 °C in a Spinco No. 50 fixed angle rotor. The pellets were resuspended and washed three times with 10 mM Tris–HCl, pH 7.4. The quantities of hemoglobin found to be membrane-bound were similar with each of these separation procedures. After the second wash, very little radioactivity was released from membranes which had been incubated with labelled hemoglobin.

Electron microscopy

Pellets of membrane preparations were fixed in 3 % glutaraldehyde in 0.067 M phosphate buffer at pH 7.35 for 3 h and then post-fixed for 30 min in 2 % OsO_4 (Dalton's fixative). Dehydration was performed in a graded series of alcohols. Epoxy resin was used for the embedding. Ultrathin sections for electron microscopy were obtained with a diamond knife and a Porter Blum MT 2-B ultramicrotome. An Elmiskop 1A was used for electron microscope scanning.

Other procedures

Proteins were determined by the method of Lowry et al. [12]. The amount of hemoglobin remaining on the original membrane preparations was measured by the micro-method utilizing benzidine [13].

RESULTS

Preparations of normal erythrocyte membranes retained about 10 μg of hemoglobin per mg membrane protein. When these membranes were incubated with ^{14}C-labelled hemoglobin and then separated from free hemoglobin by centrifugation on a linear sucrose gradient, they were found to be capable of binding fresh hemoglobin. As shown in Fig. 1a, membranes are recovered in a single absor-

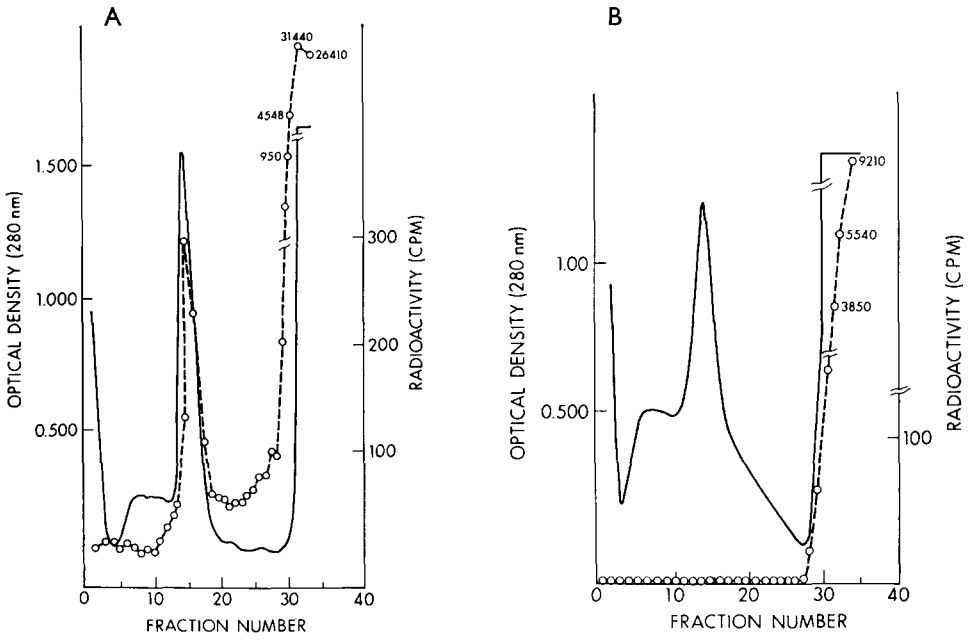

Fig. 1. The separation of erythrocyte ghosts and ^{14}C-labelled hemoglobin by sucrose gradient centrifugation. The effect of pre-incubation with hemoglobin. Normal erythrocyte ghosts were incubated with or without unlabelled hemoglobin and reincubated with labelled hemoglobin for 30 min. Aliquots were layered on preequilibrated linear sucrose gradients and centrifuged in the SW-27 rotor. The absorbance and the radioactivity were determined as described under methods. A. Erythrocyte ghost membrane (237 μg of protein) incubated with ^{14}C-labelled hemoglobin S (863 μg of hemoglobin of 73 498 dpm/mg specific activity). —, absorbance; ○—○, radioactivity. B. Erythrocyte ghost membranes pre-incubated with 420 μg of hemoglobin A in the complete incubation solution (see text). The membranes were re-isolated after 30 min of incubation by high-speed centrifugation, as described under Materials and Methods. The pre-incubated membranes (1.62 mg) were suspended in the incubation media and 711 μg of ^{14}C-labelled hemoglobin A was added followed by a second incubation period (30 min). Absorbance and the radioactivity were obtained as described under Materials and Methods. The specific activity of the ^{14}C-labelled hemoglobin A was 26 423 dpm/mg protein.

bance peak layered between 37.5 and 40 % sucrose, and are separated by 10 fractions from the soluble proteins. Preliminary experiments demonstrated that membranes which were not incubated layered at the same density as those incubated, indicating that the incubation procedure resulted in no alteration in the density of membrane vesicles. When labelled hemoglobin alone was run on the gradients, no radioactivity was found in the region where membranes banded.

By electron microscopy (Fig. 2) the membranes appeared as large, flattened vesicles. Cross section revealed the unit membrane pattern with double dense lines separated by a space. The thickness of the fixed membranes was approximately 80–90 Å in the three samples. Granules were seen attached on both sides of the membrane in all of the samples, but were more abundant in membranes of irreversible sickle cells and Fraction II preparations. The granules were ill-defined with fuzzy contour and were larger than ribosomes. The question of whether they really represent hemoglobin granules attached to the membranes, or whether they are the

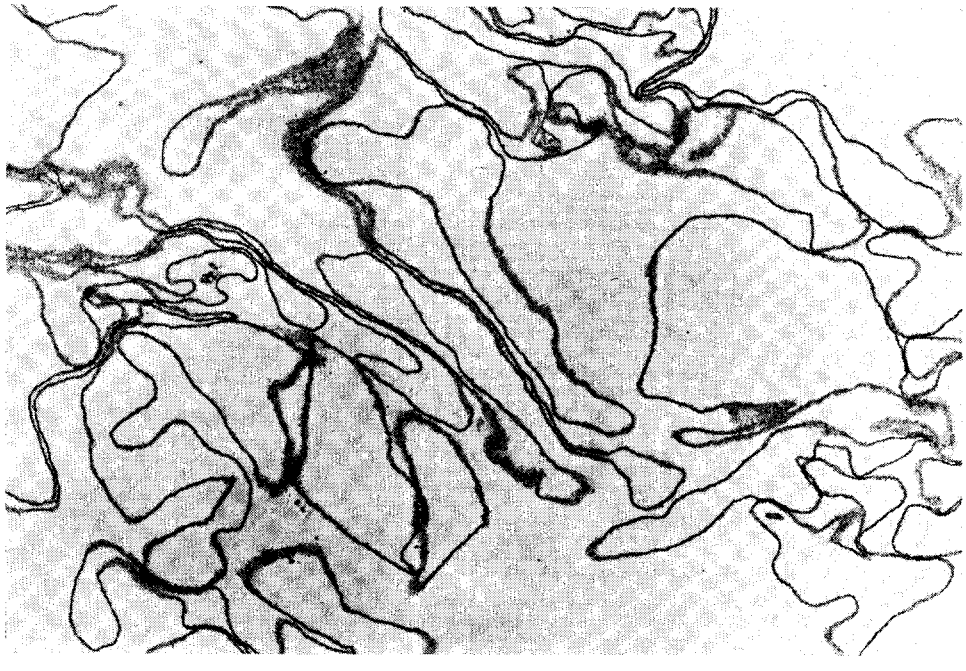

Fig. 2. Purified membranes of sickle red blood cells (Fraction II). This electron micrograph shows the richness of the preparation. The membranes form long, flattened vesicles. Areas of fuzziness correspond to tangential section of the membrane. (Magnification: 10 000 ×.)

product of the staining and contrasting techniques used for electron microscopy, should remain open and await further examination.

When membranes were preincubated with unlabelled hemoglobin, isolated by centrifugation, and reincubated with labelled hemoglobin, very little radioactivity was associated with the membrane peak (Fig. 1b), suggesting that maximal binding took place during the first incubation, and that loss of bound hemoglobin during the isolation procedure and exchange between free and bound hemoglobin during reincubation were both minimal.

The effect of some variations in the composition of the incubation media and in conditions of the incubation on membrane binding of hemoglobin are shown in Table I. Elimination of cations (Mg^{2+} and K^{+}) from the media resulted in more than a 50 % decrease in hemoglobin binding. It should be pointed out that from one preparation to another, variability of hemoglobin binding of the order of 15 or 20 % was observed.

The finding of only a moderate decrease in hemoglobin binding with omission of the sulfhydryl reagent dithiothreitol suggests that reduction of –S–S– bonds of the membrane plays a minor role in its hemoglobin binding capacity. Varying the temperature of the incubation from 4 to 22 °C produced no change in the quantity of hemoglobin bound, suggesting that hydrophobic interactions are of little importance in the binding phenomena. No differences in binding were found when the time of incubation was varied between 10 and 120 min.

As shown in Fig. 3, addition of increasing quantities of ^{14}C-labelled hemoglo-

TABLE I

THE INCUBATION CONDITIONS FOR MEASURING THE BINDING OF HEMOGLOBIN TO ERYTHROCYTE PLASMA MEMBRANES

The incubation conditions are: In a total volume of 2.0 ml the final concentration, in mM: Tris–Cl, 100 (pH 7.4); dithiothreitol, 10; $MgCl_2$, 10; KCl, 10. Incubated for 30 min at 2 °C, unless otherwise stated. At the end of the incubation time an aliquot was layered on preequilibrated sucrose gradient and run in the SW-27 at 20 × K for 120 min at 4 °C. Samples were collected and the absorbance obtained through an automatic recording spectrophotometer. Afterwards the proteins were precipitated with trichloroacetic acid and processed for the determination of the radioactivity as described under Materials and Methods. The amount of membrane protein was 1400 μg, and the labelled hemoglobin had a specific activity of 26 423 dpm/mg of hemoglobin. 797 μg of hemoglobin were added to each tube.

Incubation system	$\frac{\mu g \text{ hemoglobin bound} \times 10^2}{\mu g \text{ membrane protein}}$	Recovery (%)
Complete system	1.19	100
Complete system $-(Mg^{2+}+K^{2+})$	0.57	48.5
Complete system −dithiothreitol	0.74	62
Complete system at 22 °C	1.16	97
Complete system incubated for 60 min	1.13	94

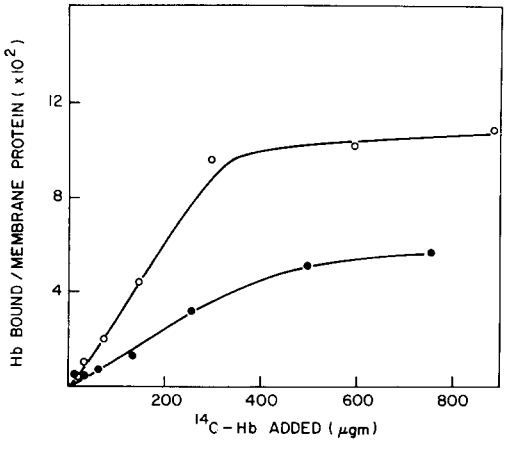

Fig. 3. Binding of increasing amounts of hemoglobin A and hemoglobin A_2 to erythrocyte membranes. A constant membrane amount of 148 μg was utilized in these experiments. The hemoglobin A addition varied between 12.6 and 757 μg and the hemoglobin A_2 between 14.9 and 984 μg. At the end of the incubation time the membranes were recovered by centrifugation. From the membrane pellet the trichloroacetic acid-precipitable radioactivity was obtained as described under Materials and Methods. The specific activity of hemoglobin A was 35 000 dpm/mg of hemoglobin and for hemoglobin A_2 46 261 dpm/mg hemoglobin. ●—●, hemoglobin A; ○—○, hemoglobin A_2. Ordinate: μg of hemoglobin bound per μg of membrane protein amplified 10^2 times. Abcissa: amount of hemoglobin added during incubation.

bin to a constant amount of membrane (protein) resulted in a progressive increase in the amount of hemoglobin bound to the membrane until a saturation level was reached. The saturation level for hemoglobin A under these conditions was about 52–58 μg

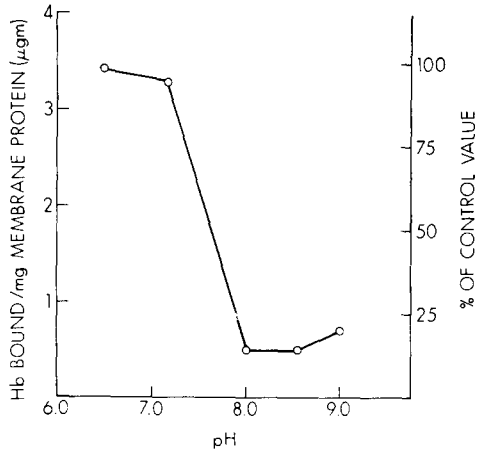

Fig. 4. The effect of pH on the binding of hemoglobin A to normal erythrocyte ghosts. The incubation solution was as described in Table I, with the following modifications; for the tubes at pH 6.5, 100 mM phosphate buffer, was used. For the remaining points the Tris–HCl buffer was adjusted accordingly. The amount of membrane protein was 584 μg and of hemoglobin was 194.9 μg. The specific activity of the ^{14}C-labelled hemoglobin A was 37 183 dpm/mg. Ordinate: μg of hemoglobin bound per mg of membrane protein or % of control value. Abscissa: pH.

hemoglobin per mg membrane protein, whereas with hemoglobin A_2 nearly twice this amount (112 μg hemoglobin A_2 per mg membrane protein) was bound.

The effects of varying the pH of the incubation media are shown in Fig. 4. There was virtually no difference in hemoglobin between pH 6.5 and the standard conditions of pH 7.35. But when the pH was elevated to 8.0 or higher, hemoglobin binding was drastically reduced to less than 20 % of that found at pH 7.35.

Hemoglobin binding was found to be disrupted at high ionic strength. When, after the standard incubation, the concentration of NaCl in the incubation mixture was raised to 0.5 or 1.0 M and the usual resuspensions and washing procedures with 10 mM Tris buffer were performed (see alternative separation procedure in Materials and Methods), almost 90 % of bound hemoglobin was released from the membranes.

As shown in Fig. 5, the dense lower fraction (Fraction I) of homozygote S red cells, in which the proportion of irreversibly sickled cells was greatly enriched, retained three times more hemoglobin than did normal red cells when membranes were prepared by hypotonic lysis. The ability of these Fraction I membranes to bind new homoglobin was found to be considerably less than that of normal membranes of the other homozygote S fractions: after incubation, the amount of new hemoglobin bound by Fraction I membranes was one third that of the normal ghosts. In contrast, the middle and upper fractions of homozygote S cells (Fractions II and III), whose residual hemoglobin was only slightly greater than that of the normal ghosts, bound nearly as much new hemoglobin as did the normal membranes. It should be noted that the irreversible sickle cell-rich fraction, Fraction I, comprised only about 8 % of the volume of homozygote S red cells. Thus it was not surprising that membranes prepared from unfractionated homozygote S blood showed a hemoglobin-binding capacity similar to that of normal membranes.

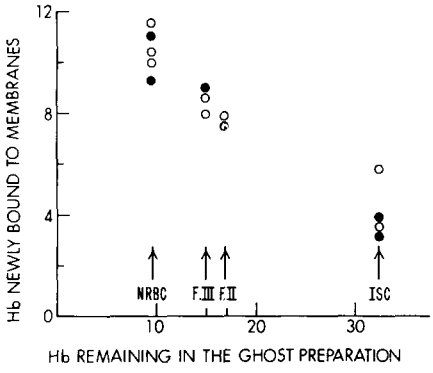

Fig. 5. The binding of hemoglobin to membranes isolated from normal and free fractions of sickle cells Abscissa: Hemoglobin remaining in the ghost preparation as μg of hemoglobin per mg of membrane protein. Ordinate: Newly bound hemoglobin to the membranes, also expressed as ng of hemoglobin per ng of membrane protein. NRBC, normal red blood cell; FIII and FII, Fractions I and II; ISC, irreversible sickle cells. Filled circles: Hemoglobin S; open circles: Hemoglobin A. Conditions of the experiment were: Membrane protein used was approximately 500 μg, and about 100 μg hemoglobin was added to each tube. Rest of the conditions as in Table I.

At low membrane protein to hemoglobin ratios at which these experiments were performed, no difference was observed between hemoglobin A and hemoglobin S binding. Furthermore, for each of the membrane preparations from normal and homozygote S cells, including the irreversible sickle cell-rich Fraction I, the amount of

TABLE II

RED CELL MEMBRANE BINDING COMPETITION EXPERIMENTS WITH HEMOGLOBIN A, S AND A_2

The incubation was performed in 0.7 ml as described in Table I. The membrane bound ^{14}C-labelled hemoglobin was obtained after trichloroacetic acid precipitation of the three times washed membranes, as described under Materials and Methods. Hemoglobin A had 35 000 dpm/mg hemoglobin; hemoglobin A_2 46 261 dpm/mg hemoglobin and hemoglobin S had 73 500 dpm/mg hemoglobin. Since the hemoglobin:membrane protein ratios varied in different groups of experiments, numerical comparisons between the groups are approximate.

Mixtures	$\frac{\mu g\ ^{14}C\text{-hemoglobin bound}}{\mu g\ \text{membrane protein}} \times 10^2$
Hemoglobin A*	1.33
Hemoglobin S*	2.48
Hemoglobin A_2*	9.45
Hemoglobin A* + hemoglobin S	1.01
Hemoglobin A + hemoglobin S*	1.81
Hemoglobin A* + hemoglobin A_2	0.94
Hemoglobin A + hemoglobin A_2*	7.37
Hemoglobin S* + hemoglobin A_2	3.05
Hemoglobin S + hemoglobin A_2*	5.60

* Hemoglobin, labelled with ^{14}C.

hemoglobin bound was similar when incubated with large concentrations of hemoglobin A or with hemoglobin S.

The results of binding experiments which compare the relative affinities of hemoglobin A, hemoglobin S and hemoglobin A_2 for red cell membranes are shown in Table II. Mixtures of approximately 100 μg each of two hemoglobin components were incubated with 148 μg of red cell membrane protein. Incubations in which one of the components of the mixture was labelled with ^{14}C-labelled nitrogen mustard were compared with incubations in which the complementary variant was labelled. By determining the amount of labelled hemoglobin bound to the membrane protein, the relative affinity of these variants for red cell membranes was determined.

When 148 μg of red cell membrane were exposed to approximately 60 μg of either hemoglobin A, S or A_2, there was progressively more hemoglobin bound to the membranes in the order listed, which corresponds to an order of increasing net positive charge of the hemoglobin.

DISCUSSION

The experiments described utilize a system in which the actual binding of native hemoglobin to a red cell membrane preparation can be observed and measured. With this system, the effects of pH, temperature, ionic strength and of a single point mutation (hemoglobin S) on this binding process have been observed.

Before drawing conclusions from these data, the limits of the observation should be clearly defined. These findings refer, first, to a membrane preparation method which involved hypotonic solution no lower than 22 mosM. It has been clearly demonstrated that one of the variables which determines the amount of hemoglobin remaining in the final preparation and the composition of the membranes is the ionic strength of the final wash [3, 14]. Secondly, measurements were made on freshly drawn blood, as different results might be obtained if cells allowed to age "in vitro" are used, Finally, these studies were conducted with hemoglobin alkylated with ^{14}C-labelled nitrogen mustard. We have previously shown that hemoglobin function, as judged by oxygen affinity and Bohr effect, is unaltered after alkylation [11]. Hence, no significant conformational changes of oxyhemoglobin would be expected. In addition, with prolonged dialysis after the reaction with nitrogen mustard, the net charge and the solubility of the hemoglobin is the same as that of native hemoglobin. While polymer formation of deoxyhemoglobin S is inhibited by nitrogen mustard, these studies utilized only oxyhemoglobins.

The data presented demonstrate that red cell membranes are capable of binding native hemoglobin (as the preliminary work of Mitchell et al. [4] seems to indicate) and provides some information about the nature of some of the bonds involved in hemoglobin–membrane interactions. The absence of inhibition of the binding at low temperatures argues against a significant or major role of hydrophobic bonds on the binding. Moreover, the strong pH dependence of the process (drastic decrease in binding above pH 7.3) and the release of hemoglobin almost quantitatively by washing the incubated membranes with 0.5 or 1.0 M NaCl, suggest that electrostatic bonds are an important participant in the binding. Nevertheless, the possibility of high ionic strength solubilizing membrane protein may be a contributing factor [15]. These characteristics are in accordance with previous observations on the conditions for the retention of hemoglobin in red cell ghost preparations [4, 14].

Membranes prepared from red cells of persons homozygous for hemoglobin S retain significantly more hemoglobin than membranes prepared from normal cells. Moreover, this increased retention of hemoglobin is attributable entirely to the dense fraction of homozygote S red cells, which contains a high proportion of irreversibly sickled cells. The upper fractions of homozygote S red cells, which have very few irreversible sickle cells, retain as little hemoglobin after the final wash as do normal red cells. These findings are consistent with the observations of Lessin [16] that molecular aggregates (which he suspected were hemoglobin molecules) are associated with the internal aspect of the membranes of homozygote S red cells. Thus, it appears that the increased association of hemoglobin S with the red cell membrane preparation is a function of the conversion of the red cell to an irreversible sickle cell. In other words, one aspect of irreversible sickle cell development seems to involve an alteration of the membrane structure which enhances considerably the extent of binding of hemoglobin to the membrane.

Hemoglobin S has been found to associate preferentially with membrane preparations. Klipstein and Ranney [17] observed that hemoglobin associated with membrane preparations from hemoglobin S trait and from hemoglobin C trait individuals contained a higher proportion of hemoglobin S and hemoglobin C, respectively, than expected from the hemoglobin composition of the cells; normal red cell membranes retained a higher proportion of hemoglobin A_2 than the hemolysate. One possible explanation of these findings was that the different net charges of these hemoglobins was related to their different affinities for the membrane. We have tested this hypothesis in the present system, and the results depicted in Table II demonstrate that hemoglobin A_2 does indeed have a higher affinity for the membrane than hemoglobin A, and that hemoglobin S has an intermediate affinity in comparison with the other two. These results should only be interpreted in relative terms because the extent of binding of pairs of hemoglobins may have complex interrelationships.

The data presented here complements and extends the studies performed on the retention of hemoglobin in red cell ghosts [4]. We have established that red cell membrane can indeed bind hemoglobin, and that this binding is dependent upon the pH and ionic strength of the media. In addition, hemoglobin molecules attach themselves to the membrane in direct proportion to their electropositivity. Finally the data indicate that the irreversible sickle cell fraction of sickle cell anemia blood is primarily responsible for the increased retention of hemoglobin in homozygote S membranes.

The interaction of hemoglobin molecules with red cell membranes may be only one instance of a more general phenomenon: the participation of soluble intracellular proteins in the dynamic structure of the cell membrane; such interactions may in turn alter some of the structural and functional properties of the bound proteins, resulting in separate physiological functions or in pathological consequences.

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